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Investigations into the absorption of insulin and insulin derivatives from the small intestine of the anaesthetised rat

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ABSTRACT

Experiments have been undertaken to determine the extent to which cholic acid conjugates of insulin were absorbed from the small intestine of anaesthetised rats by means of the bile salt transporters of the ileum. The measure used to assess the absorption of the cholyl-insulins was the amount of hypoglycaemia following infusion into the small intestine. Control experiments involving infusion of natural insulin into the ileum showed either nil absorption or absorption of a small amount of insulin as indicated by transient dip in the blood glucose concentration. However, when insulin was co-infused with the bile salt taurocholate, this was followed by a marked hypoglycaemic response which was specific to the ileum and did not occur on infusion into the jejunum. When the two cholyl conjugates of insulin were tested *viz.* B^{29}-Lys-cholyl-insulin and B^{1}-Phe-cholyl-insulin, both were biologically active as indicated by hypoglycaemic responses on systemic injection, though their potency was about 40% of that of natural insulin. While there was no evidence for the absorption of B^{29}-Lys-cholyl-insulin when infused into the ileum, B^{1}-Phe-cholyl-insulin did cause a long lasting hypoglycaemic response, indicating that absorption had occurred. Since the hypoglycaemic response was blocked on co-infusion with taurocholate and was absent for infusion of the conjugate into the jejunum, these results were taken as evidence that B^{1}-Phe-cholyl-insulin had been taken up by the ileal bile salt transporters. This would indicate that B^{1}-Phe-cholyl-insulin is worthy of further investigation for use in an oral insulin formulation.

1. Introduction

Since its discovery in 1922, the hormone insulin has been administered by sub-cutaneous injection for the treatment of diabetes mellitus (Type 1) which arises from the loss of the β-cells of the Islets of Langerhans of the pancreas and the subsequent loss of insulin production [1]. The diabetic patient suffers not only from uncontrolled blood glucose levels but also, in the longer term, from vascular disease due to glycosylation of haemoglobin. To this end, more intensive therapy involving at least 3 injections of insulin each day has been shown to be beneficial in reducing the incidence of retinopathy and nephropathy [2]. Compliance can, however, be compromised by an aversion to self-injection due to discomfort, abscess formation, scarring and lipohypertrophy at the site of injection [3]. Hence, alternative methods of treatment in the form of power injection of insulin into the skin with pressurised helium gas [4], through inhalation of fine particles of insulin for absorption from the alveoli [5] and through pancreatic islet cell transplantation [6] have been developed, though none of these has proved to be a successful replacement for insulin injections. The most desirable method of delivery would be an oral formulation taken at meal-time, though this is fraught with several major considerations. First, there is the need for protection of the orally-ingested insulin against digestion by gastro-intestinal proteases [7, 8, 9, 10]. Then, once delivered into the small intestine, there then arises the not inconsiderable problem of promoting the absorption of such a large charged molecule which consists of an A chain of 21 amino acids joined by 2 disulphide bridges to a B chain of 30 amino acids, across the intestinal mucosa into the mucosal circulation. This would have to occur repeatedly for several applications of the formulation per day, extending over the individual's life time, without the risk of cumulative damage to the intestinal mucosa. Of critical importance would be the quantity and the timing of ingestion of the formulation relative to the meal to be consumed. Long term safety in terms of the toxicology of the insulin delivery system and the innocuousness of non-absorbed insulin in the colon would also need to be assured [11]. While an oral insulin formulation is beset with many difficulties, there have nevertheless been numerous efforts over the past decades towards reaching this goal. The consensus is that absorption of insulin from the small intestine, when infused in a moderate dose in a saline vehicle, does not occur to any significant extent in animal models [12, 13, 14, 15, 16, 17, 18 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34], though some uptake has been reported for jejunal and ileal infusions in conscious rabbits [35] and with high luminal doses in rabbit [30] and in rat [26, 36]. In the promotion of insulin uptake, the approaches can be divided broadly into the promotion of delivery of natural insulin to maximise its concentration at the small intestinal mucosa and through the uptake of covalently-bonded insulin.

1.1. Promotion of absorption of natural insulin

There have been numerous investigations involving different insulin formulations with the purpose of promoting the absorption of natural insulin from the small intestine, as shown in the summary presented in Table 1. In addition, there are also published results of clinical trials which do not impart specific details of the formulation adopted *e.g.* ORMD-0801 [37] and there is some limited information available from the web sites and press releases of pharmaceutical companies [38]. In general, the published results for the different insulin carrier systems, with one exception in which insulin uptake was reported only from the colon [33], have indicated considerable success. This is exemplified by the outcome of the 36 studies involving animal models in which infusion of the insulin formulation into the small intestine *in vivo* led to a reduction in the blood glucose concentration by 49 $\pm$ 3 % with a duration of action of 3.4 $\pm$ 0.4 h (mean $\pm$ S.E.M.).

The routes by which insulin is absorbed across the small intestinal mucosa may be transcellular or paracellular. The small intestinal epithelium consists of about 90% enterocytes and 10% goblet cells which secrete mucus. These are joined at their luminal border by tight junctions which consist of interleaving loops of the protein occludin which can be regulated to increase the pore diameter of the junctions [39]. In the ileum, there are also M cells (membranous epithelial cells) which overlie the Peyer's patches and which are known to be able to take up macromolecules from the lumen [40]. Immunocytochemical studies suggest that the major route for absorption of free insulin is by the transcellular route through the enterocytes [41] in which macromolecules are taken up at the luminal membrane by phagocytosis or pinocytosis followed by exocytosis at the basolateral membrane and absorption into the mesenteric circulation [42]. This route of absorption is facilitated by co-infusion of insulin with cell-penetrating peptides [23, 43, 44] and bile salt liposomes [45]. In addition, there may be uptake through pores in the mucosa which form transiently as a consequence of the shedding of the enterocytes at the tips of the villi [46]. By contrast, insulin nanoparticle absorption has been described as occurring by the paracellular route through the tight junctions and intercellular clefts [47, 48, 49] and this process is enhanced by permeant inhibitors of phosphatase [31]. However, it has been claimed that, while nanoparticles do provide protection against the action of intestinal proteases, there was no resulting enhancement of absorption [50]. Also, the paracellular route is not the only means of absorption since lauryl succinyl chitosan also passes transcellularly [25], while poly(-ϵ-caprolactone) is absorbed specifically by the Peyer's patches [51] and, in the case of vitamin B_{12}-linked nanoparticles, absorption would be by the vitamin B_{12} transporters which are located in the ileum [52].

Table 1

Summary of strategies to promote absorption of natural insulin

	Vehicle	Constituent	References
(i)	Saline vehicle	apoprotinin[1]	[20, 33, 34, 53]
		bile salts	[20, 33, 34, 53, 54]
		bile salts and apoprotinin	[34, 41, 53, 55]
		bile salts and bicarbonate	[54]
		bile salts and fatty acid mixed micelles	[29, 56]
		bile salts, palmitic acid, tocopherol	[19]
		caproic acid	[20]
		cetomacrogol or 5-methoxysalicylate[2]	[27, 32]
		hyaluronic acid	[57, 58]
		hyaluronidase[3]	[21]
		oligo-arginine, penetratin[4]	[23, 43, 44]
		permeant inhibitor of phosphatase	[31]
		phenylalanine derivatives	[12]
		zonulin occludin toxin[5]	[18]
(ii)	Enteric-coated	calcium phosphate	[59]
		cellulose acetate phthalate	[60, 61, 62]
		gelatin	[63]
(iii)	Liposomes	glycocholate	[24]
		red cell ghosts or liposomes	[11, 36]
(iv)	Water/oil/water emulsions	micro-emulsions	[9, 46]
		saturated or unsaturated fatty acids	[17, 30, 64]
		4CNAB[6]	[38, 65]
(v)	Nano/micro particles	chitosan	[66]
		chitosan/poly(γ-glutamic acid)	[48, 63]
		cyclodextrin polymethacrylic acid hydrogel	[28]
		hyaluron-coated fibrillar insulin	[16]
		isobutyl-2-cyanoacrylate	[67, 68]
		lauryl succinyl chitosan	[25]
		phosphatidylcholine	[69]
		poly(lactic acid)-pluronic acid	[70]
		polymethacrylic acid polyethylene glycol	[22]
		poly(-ϵ-caprolactone)	[51]
		polymerised fumaric acid and sebacic acid	[71]
		trimethyl chitosan	[49]
		vitamin B_{12}-linked dextran-epichlorohydrin	[15]
		vitamin B_{12}-linked polyallylamine	[72]

[1] protease inhibitor, [2] surfactants, [3] pretreatment to degrade glycocalyx overlying mucosal epithelial cells, [4] cell-penetrating peptides, [5] to open tight junctions, [6] monosodium N-(4-chlorosalicyloyl)-4-aminobutyrate

1.2. Absorption of covalently-bonded insulin

Investigations involving the absorption of covalently-bonded molecules have consisted of the following.

(i) Insulin has been conjugated at its B^1 -NH_2 group or at both the B^1 and B^{29} NH_2 groups with palmitic acid [73], caproic acid [13] and methoxy-polyethylene glycol (PEG) [14] in order to increase lipid solubility in the enterocyte luminal membrane.

(ii) Conjugation of customised peptides at the C^3 hydroxyl group of cholic acid has been made with the purpose of promoting uptake by the bile salt transporters which are located in the ileum [74]. This, however, was successful only for peptides of up to ten amino acids [75]. Conjugation has also been undertaken at the C^{24} carboxyl group *via* a γ glutamyl link, though uptake was limited to tetrapeptides and hexapeptides [76].

(iii) Direct conjugation to the C^{24} carboxyl group of cholic acid has been undertaken with triiodothyronine, though the purpose in this case was to reduce the uptake by the heart since triiodothyronine is already absorbable from the small intestine [77]. Successful absorption of a carbohydrate molecule in the form of heparin which was conjugated at an amine group to the C^{24} carboxyl group of deoxycholic acid has also been reported [78].

In our experiments into the absorption of gastro-intestinal hormones from the small intestine of anaesthetised rats, we conjugated the hormone directly to the C^{24} carboxyl group of cholic acid. Conjugation was made directly to the N terminus of the 34 amino acid hormone gastrin which stimulates gastric HCl secretion [79] and the 27 amino acid hormone secretin which stimulates pancreatic HCO_3^- secretion [80]. Conjugation had the effect of increasing the biological activity of gastrin while that of secretin was reduced. However, in both cases, appreciable absorption of the cholyl-gastrin and cholyl-secretin was recorded with the latter achieving a bio-availability of 20-37% [80]. Since the uptake was specific to the ileum and did not occur from the jejunum and since it was blocked by co-infusion with taurocholate which was used as a competitive inhibitor, we concluded that the cholyl hormone derivatives had been absorbed by the bile salt transporters. It is this approach which we now extend to the absorption of cholyl-insulin. In the initial experiments, we investigated the A21,20-S-S-B19-26 decapeptide of insulin and its B^{19}-Cys-cholyl-derivative since these were amenable to chemical synthesis and the insulin decapeptide has been reported to possess 77% insulin activity [81]. However, the decapeptides proved to be devoid of biological activity and, so, we then tested the extent to which there was absorption of the cholyl derivatives of the full insulin molecule which possesses three free -NH_2 groups. Since the A^1-Glycine residue is required for hormone activity [82], we tested the absorption of, first, B^{29}-Lys-cholyl-insulin and, second, B^1-Phe-cholyl-insulin. Experiments were also undertaken to assess the absorption of natural insulin in order to provide a reference for the cholyl-insulin experiments.

2. Materials and methods

2.1. Surgical procedures

Experiments were carried out on male Wistar rats, weight 240-360 g in accordance with the Animals (Scientific Procedures) Act 1986. The animals were fasted overnight but were allowed a 5% sucrose drink in addition to water. They were anaesthetised with an intra-peritoneal (I.P.) injection of pentobarbitone sodium (Rhône Mérieux, Harlow, UK) at a dose of 80 mg kg^{-1}. The criterion for anaesthesia was abolition of the hind limb flexor withdrawal reflex. In order to maintain the level of anaesthesia, supplementary I.P. injections (24 mg kg^{-1}) were given as required. At the end of the experiments, the animals were killed with an intra-venous (I.V.) or intra-arterial (I.A.) injection of 100 mg Euthatal (Merial Animal Health, Harlow, UK).

The initial surgical procedures consisted of tracheostomy to allow tracheal aspiration and artificial ventilation, if necessary, and cannulation of the carotid artery with a Portex cannula of outer diameter 1.0 mm to monitor the arterial blood pressure. This was followed by cannulation of the external iliac vein with a cannula of outer diameter 1.0 mm or of the external iliac artery with a cannula of outer diameter 0.75 mm in order to allow blood sampling or injection of hormones. The cannulae contained heparinised saline at 100 I.U. heparin ml^{-1} (Leo Laboratories Ltd., Princes Risborough, UK). After a midline laparotomy, the pyloro-duodenal junction was ligated to prevent entry of gastric contents into the duodenum and hence the possible release of duodenal hormones. The terminal ileum was cannulated with a perforated Portex cannula of 2.0 mm outer diameter which extended from a point of entry in the wall of the proximal colon through the ileo-colic junction into the ileum. The ileum was then gently infused under visual observation with 0.5 mL saline solution at 37°C, which was followed by slow withdrawal to remove any bile and residual digestive matter. This process was repeated until the effluent was clear. In some cases, an outlet cannula of 4 mm outer diameter was inserted 15 cm from the inlet cannula to facilitate flushing. When jejunal cannulation was required, this was undertaken with a perforated Portex cannula of 2.0 mm outer diameter with a point of entry just distal to the ligament of Treitz. In these cases, the small intestine was also ligated *ca.* 30cm from the ileo-colic junction to prevent entry of the jejunal infusate into the ileum. The jejunum was also flushed with warm saline solution to remove the luminal contents. Some animals had both ileal and jejunal loops. Finally, bilateral cervical vagotomy was undertaken to remove vago-vagal reflexes. The animal's temperature was maintained as near as possible to 37°C and, at the end of the experiment, samples of the ileal/jejunal loops were taken for histology.

2.2. Blood samples

Blood glucose measurements were made in duplicate, with further samples taken if the duplicates differed appreciably, using the Accu-Chek glucose meter (Roche Diagnostics, Burgess Hill, UK) which was calibrated with the manufacturer's calibration solutions. Blood samples were obtained initially from the external iliac artery or vein and then by incision of the tail artery. In the former, 2 small samples of 0.2 mL blood were withdrawn and the second sample was assayed: unused blood was then returned followed by a small saline flush. In the latter, the first drop of blood was removed and the second drop assayed. The incision was then sealed with surgical tape. In 4 experiments, sufficient blood was withdrawn systemically to allow a parallel photometric assay using glucose oxidase and dianisidine (Sigma GAGO-20,; Sigma, Poole, UK). For 40 samples with a range of glucose concentrations of 0.5 to 9 mM, there was strong correlation between the two assays ($r = 0.96,\ P < 0.001$), which confirmed the suitability of the Accu-Chek glucose meter. In 7 experiments, one of which is shown in Fig. 1, comparisons were made between parallel Accu-Chek glucose readings covering a range of 2 to 9 mM taken from 21 pairs of deep arterial and tail artery samples. These also showed a highly significant correlation ($r = 0.99,\ P < 0.001$) and confirmed the suitably of tail artery sampling which had the advantage of causing less disturbance to the arterial blood pressure.

2.3. Injections and infusions

I.A. or I.V. injections of hormones were made with the hormone dissolved in 0.1 mL saline and were followed by 0.2 mL saline to flush through the cannula. For intra-ileal or intra-jejunal infusions, the appropriate weight of hormone was dissolved in 1.0 ml saline which was either phosphate buffered saline (147 mM NaCl, 3.0 mM Na_2HPO_4) or citrate-phosphate saline (144 mM NaCl, 3.0 mM Na_2HPO_4, 1.0 mM citric acid) with the purpose of chelating any zinc ions to preclude the possibility of hexamer formation [83]. The pH of the saline was set to 8.2 at a temperature of 37°C and was infused slowly over a period of 3 min when it was followed by a wash-in infusion of 0.2 ml saline. In control experiments, slow infusions of 1.0 mL saline at pH 8.2 were also made. The rate of absorption of fluid from the intestinal loop was also monitored by visual inspection at 30 min intervals. In some experiments, co-infusions were made with 20 mM sodium taurocholate (Sigma T-4009) which is the main bile salt in the rat [84], with the dose of 20 mM being at the lower end of the normal range in natural bile [85]. Protease inhibitors were not used in the experiments.

2.4. Insulin conjugates

Since insulin is a highly conserved hormone with only small differences between species [86], we were not constrained by the source of the insulin and control experiments were made with human recombinant insulin, molecular weight (MW) 5808 (Sigma I-9278). Cholyl-insulin, MW 6124, was manufactured by the conjugation of bovine insulin (Sigma I-5500) to cholic acid *N*-hydroxysuccinimide ester. Dimer and hexamer insulin self-association was prevented by use of a citrate buffer. Conjugation with the C^{24} carboxyl group of cholic acid was achieved at the B^1-Phe NH_2 residue by setting the insulin buffer solution at pH 8.5 and at the B^{29}-Lys NH_2 residue with pH 10. Addition of small aliquots of cholic acid-*N*-hydroxysuccinimide ester to the bovine insulin was followed by the appropriate aliquots of NaOH solution which was used to maintain the pH throughout the conjugation reaction. When an analytical hplc indicated the optimum reaction yield of mono-conjugated insulin, the reaction was stopped and the cholyl-insulin precipitated by acidification with HCl to pH 2-3. Preparative reversed-phase hplc was employed to recover pure mono-conjugate and remove unconjugated and multiple conjugated insulin. The purified material was freeze-dried and stored at -20°C. Insulin decapeptide (I10)

(A21,20-S-S-B19-26, MW 1210)) and cholyl-insulin decapeptide (B^{19}-Cys-cholyl-I10, MW 1600) were synthesised *de novo* by polymer-supported peptide synthesis techniques [79]. The MW of I10 and the cholyl-insulins was confirmed by mass spectrometry (MS) and identification of the cholate binding site was undertaken by MS analysis of the products of trypsin digest of the cholyl-insulin molecules.

2.5. Histology

As a standard procedure in the intestinal infusion experiments, a 5 cm length of small intestine was resected immediately after termination of the experiment, infused with 10% formal saline and immersed in 10% formal saline overnight. The tissue was then processed for paraffin embedding and transverse sections were made at several locations in each segment of gut. These were stained with haematoxylin/eosin and alcian blue for light microscopy.

2.6. Data analysis

In addition to representative experiments from single animals, group results are presented for the intestinal infusion experiments in the form of the mean ± S.E.M. normalised to a control blood glucose concentration of 5.0 mM. Following an experimental procedure, the hypoglycaemic response was measured as the decrement in the blood glucose concentration from the preceding baseline for each 15 min test period over the duration of the response. If, instead of a level baseline, there was progressive hyperglycaemia, *e.g.* Fig. 1, the response was obtained as the decrement from the best fitting regression line derived from data values which occurred prior and subsequent to the response. An overall response- the cumulative response- was then calculated as the summation of the decrements at each 15 min test period. Tests against zero were made with the t-test while comparisons of results between cohorts of animals were made with the two sample t-test and within cohorts of animals with the paired t-test. Linear regression analysis was undertaken to compare two sets of variables. The analyses were undertaken with Minitab 15 (Minitab UK, Coventry, UK) and statistical significance was taken as $P < 0.05$.

3. Results

The present results were obtained from experiments of duration 6-8 h from a total of 114 rats in which the mean blood glucose concentration at the commencement of the experiments was 5.1 ± 0.10 mM (mean ± S.E.M.). The abbreviations which have been employed are: I.A. intra-arterial, I.V. intra-venous, I10 insulin decapeptide, I51 natural insulin. The doses for systemic injections are given as nmol kg^{-1} while, in the specific experiments illustrated in the Figures, the dose is that which was actually injected into the animal in μg. The results are expressed throughout as the mean ± S.E.M.

The arterial blood pressure at the commencement of the experiments had mean systolic/diastolic values of 130/98 mmHg. The mean arterial blood pressure showed a modest but significant reduction from 109 ± 1.36 mmHg at the commencement to 92 ± 1.67 mmHg at 5-8 h later ($P < 0.001$), while the corresponding values for the pulse pressure of 32 ± 0.9 mmHg and 33 ± 1.3 mmHg were not significantly different ($P = 0.45$). There was no discernible vascular action of insulin or cholyl-insulin when injected I.A. or I.V. since the arterial blood pressure remained unaffected.

3.1. Control experiments

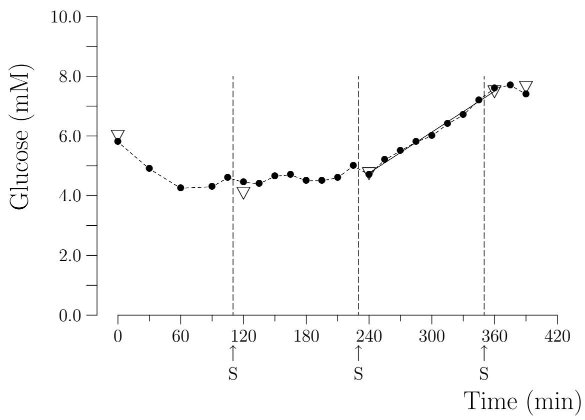

Fig. 1. Representative control experiment from a single animal showing the blood glucose levels (mM) while 3 intra-ileal infusions of 1.0 mL saline were given at 2 h intervals (S and vertical dashed lines). Blood glucose values were obtained from both the tail artery (solid circles) and the external iliac artery (inverted open triangles). The hyperglycaemic increase between 240 and 360 min has superimposed upon it the line of best fit which was highly significant ($R^2 = 99.4\%$, $P_{slope} < 0.001$).

Control experiments were undertaken in which the blood glucose concentration was followed in the absence of experimental intervention in 5 animals and when intra-ileal infusions of 1.0 mL saline were made during the course of the experiment in a further 2 animals (Fig. 1). The results were very similar in the 7 animals in that, after an initial decline to a steady level, the blood glucose concentration remained reasonably constant over the subsequent 2-3 h, after which it showed a slow progressive linear increase resulting in an elevation of the blood glucose concentration by 1-3 mM after a further 3-4

h. Fig. 1 also shows the close agreement between blood glucose concentrations obtained from sampling from the tail artery and the external iliac artery.

3.2. Systemic injections

From preliminary dose-response experiments with systemic injections of the cholyl-insulins I.A. or I.V., the minimum dose which evoked a discernible response was determined to be 1 nmol kg^{-1}. From this we set our two standard doses at 2 nmol (0.33 I.U.) kg^{-1} and 20 nmol (3.3 I.U.) kg^{-1} so as to allow the hypoglycaemic activity of the cholyl-insulins to be compared with that of natural insulin: the results are summarised in Table 2. As to be expected, systemic injection of 2 nmol kg^{-1} natural insulin, which in this case was human recombinant insulin, caused a marked hypoglycaemic response which, for 8 animals, had a mean maximum reduction of -2.9 $\pm$ 0.20 mM and a duration of $1\frac{1}{2}$ h. At the 20 nmol kg^{-1} dose, the hypoglycaemic response increased to -4.1 $\pm$ 0.31 mM with a prolonged duration which was in excess of 3 h (Fig. 2a).

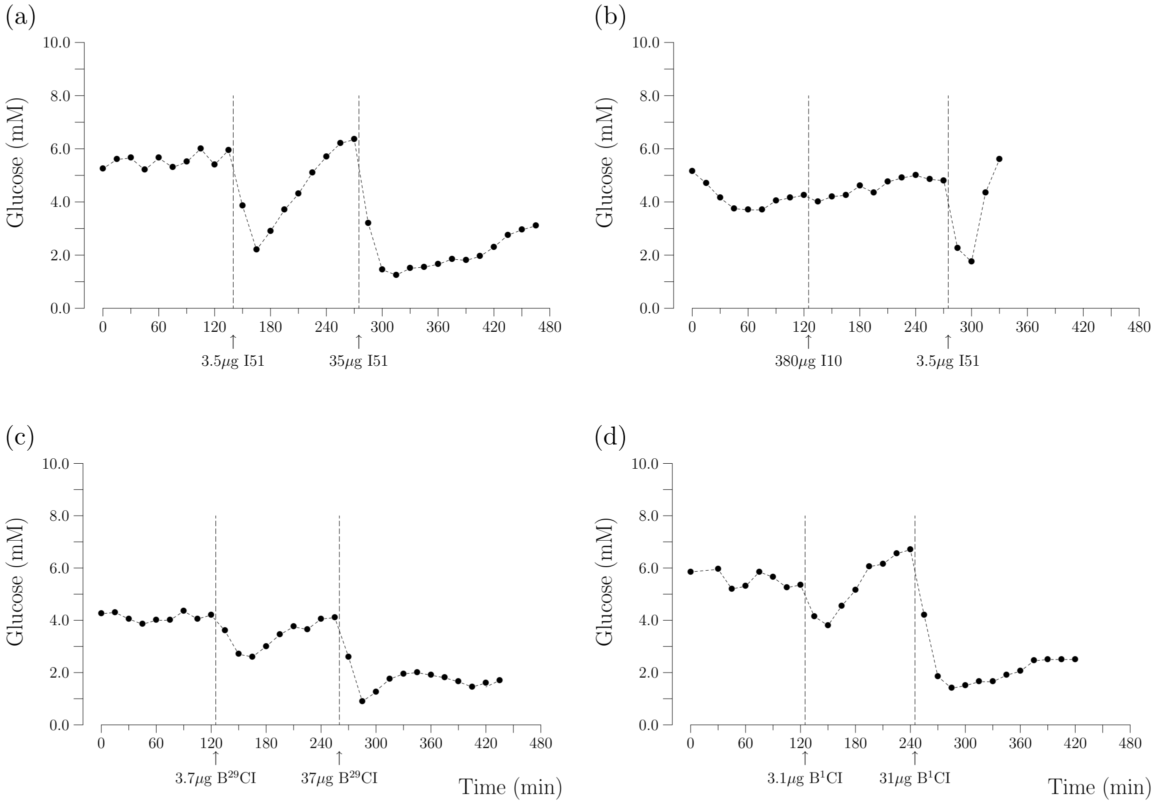

Fig. 2. Representative experiments from 4 different animals showing the effects on the blood glucose concentration (mM) of I.A. injections of (a) insulin (I51), (b) insulin decapeptide (I10), (c) B^{29}-Lys-cholyl-I51 (B^{29}CI) and (d) B^1-Phe-cholyl-I51 (B^1CI). The I.A. injections were at doses of 2.0 nmol kg^{-1} and 20 nmol kg^{-1} with the exception of I10 (b) when the dose was 1.0 μmol kg^{-1}. In (b), the responsiveness of the preparation was confirmed with an I.A. injection of 3.5 μg I51.

By contrast, neither insulin decapeptide (I10) nor cholyl-I10 showed any response to the two standard doses. Even when the dose was increased to 1.0 μmol kg^{-1} in 6 animals, there was an absence of response to both I10 and cholyl-I10. Fig. 2b shows an example of an experiment with I.A. injection of I10 and is equally representative of the outcome of the cholyl-I10 experiments. In all experiments, the responsiveness of the preparation was confirmed from the response to 2 nmol kg^{-1} insulin I.A.

Table 2

Maximum and cumulative reductions in blood glucose concentration in response to standard doses of insulin and cholyl-insulin I.A.

	n	2 nmol kg^{-1} Maximum mM	2 nmol kg^{-1} Cumulative mM	n	20 nmol kg^{-1} Maximum mM
I51	8	-2.9 ± 0.20 mM	-11.4 ± 1.3 mM	8	-4.1 ± 0.31 mM
B^{29}-Lys-cholyl-I51	6	-1.4 ± 0.22 mM**	-4.9 ± 0.6 mM**	6	-5.0 ± 0.74 mM^{+}
B^{1}-Phe-cholyl-I51	6	-1.5 ± 0.22 mM**	-4.0 ± 1.0 mM**	6	-4.2 ± 0.27 mM^{+}

Reductions in blood glucose concentration indicated by negative values of the mean ± S.E.M. Results are presented in terms of the maximum depression to each of the two doses and as the cumulative response to the lower dose. The results for each conjugate were compared to the insulin response with the two sample t-test: $^{+}P > 0.30$, $^{**}P < 0.01$.

Both the two cholic acid conjugates of the full insulin molecule, *viz.* B^{29}-Lys-cholyl-I51 and B^{1}-Phe-cholyl-I51, were biologically active, though with reduced efficacy compared to that of natural insulin (Figures 2c & d, Table 2). In the 6 experiments in which the 2 nmol kg^{-1} dose was tested, the magnitude of the hypoglycaemic response in terms of the cumulative depression of blood glucose was some 40% of that of natural insulin ($P < 0.01$). By contrast, when the dose was increased to 20 nmol kg^{-1} (6 experiments), the magnitude of the hypoglycaemic response to both conjugates increased markedly so that it became indistinguishable from that caused by natural insulin ($P > 0.30$). The response durations for both conjugates were also prolonged at this dose and were comparable to the durations for natural insulin (Fig. 2).

3.3. Intestinal infusions of insulin

Natural insulin at a dose of 0.7 mg (0.12 μmol) dissolved in 1.0 mL of phosphate buffered saline at pH 8.2 was infused into the terminal ileum of 11 animals and the effects on the blood glucose concentration was followed over several hours. These experiments were undertaken after lavage of the ileum to remove any residual bile and, in some experiments, the infusate also contained 1.0 mM citric acid to act as a Zn^{2+} chelating agent. The normalised mean data showed a transient reduction in blood glucose concentration over the period 30-75 min after infusion (Fig. 3) which was reflected in a small but significant reduction in the cumulative blood glucose concentration of -2.2 ± 0.9 mM ($P = 0.034$, Table 3). Further analysis, however, revealed 2 groups of results. In 6 animals, there was an absence of a response (Fig. 4a) as confirmed by the non-significant cumulative response of -0.3 ± 0.2 mM (P = 0.13) while in 5 animals, there was a significant cumulative response of -4.4 ± 1.42 mM (P = 0.036) which extended over 60-90 min (Fig. 4b). There was, however, no ostensible reason for this dichotomy in that both groups showed an intact mucosa under light microscopy, the time course of fluid absorption was normal with a flaccid state being reached after 2 h, and there was no difference as to whether phosphate buffered saline or citrate/phosphate buffered saline was employed. In a further 7 experiments, the intra-ileal dose of insulin was increased to 2.7 mg (0.50 μmol) with the outcome that 6 animals showed zero response while one animal showed a small hypoglycaemic dip of -1.2 mM over 30 min. Hence, overall, the outcome from these experiments is that natural insulin is not generally absorbed from the ileum though, occasionally, a small quantity may enter the systemic circulation.

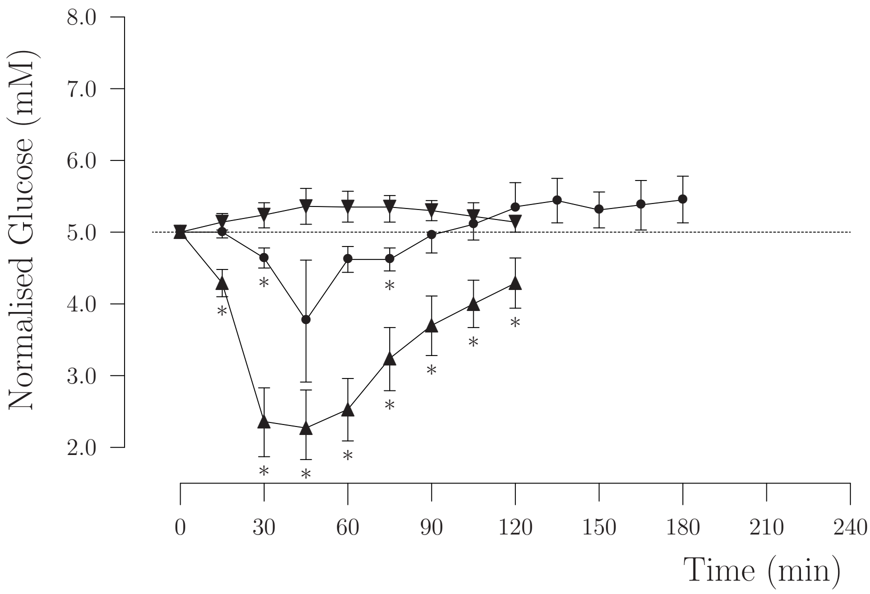

Fig. 3. Mean ± S.E.M. of blood glucose values normalised to a baseline level of 5.0 mM shown by dotted horizontal line in response to: ●-● 0.7 mg (0.12 μmol) insulin infused intra-ileally (n = 11), ▲-▲ 0.7 mg insulin with 20 mM taurocholate infused intra-ileally (n = 6), and ▼-▼ 0.7 mg insulin with 20 mM taurocholate infused intra-jejunally (n = 6). Significant reduction from 5.0 mM is denoted by * ($P < 0.05$); otherwise there was no significant difference ($P > 0.05$).

Since previous studies have indicated a facilitatory action of co-infusion of bile salts on insulin uptake [41], this was investigated using 20 mM taurocholate in phosphate buffered saline. As shown in Fig. 4c, control experiments in which taurocholate alone was infused into the ileum showed the absence of an effect on the blood glucose level (P = 0.79, Table 3). However, when 0.7 mg insulin co-infused with 20 mM taurocholate (Fig. 3, Fig. 4d, second infusion), there was a dramatic hypoglycaemic response in which the blood glucose concentration was reduced by -3.3 ± 0.9 mM, P = 0.01) with a highly significant cumulative reduction of -18.3 ± 4.2 mM (P = 0.01, Table 3). This hypoglycaemic response was specific to the ileum since no such change was recorded on infusion into the jejunum (Fig. 3, Fig. 4d, first infusion): the cumulative response of +2.1 ± 1.2 mM was non-significant (P = 0.14, Table 3). The facilitatory action on insulin absorption from the ileum also occurred at a much reduced dose of 0.5 mM taurocholate, though with a reduced response magnitude, and was also evident when prior lavage of the ileum was not undertaken. This suggests that residual bile salts in the ileum may account for the small hypoglycaemic response evident in some insulin experiments (Fig. 4b).

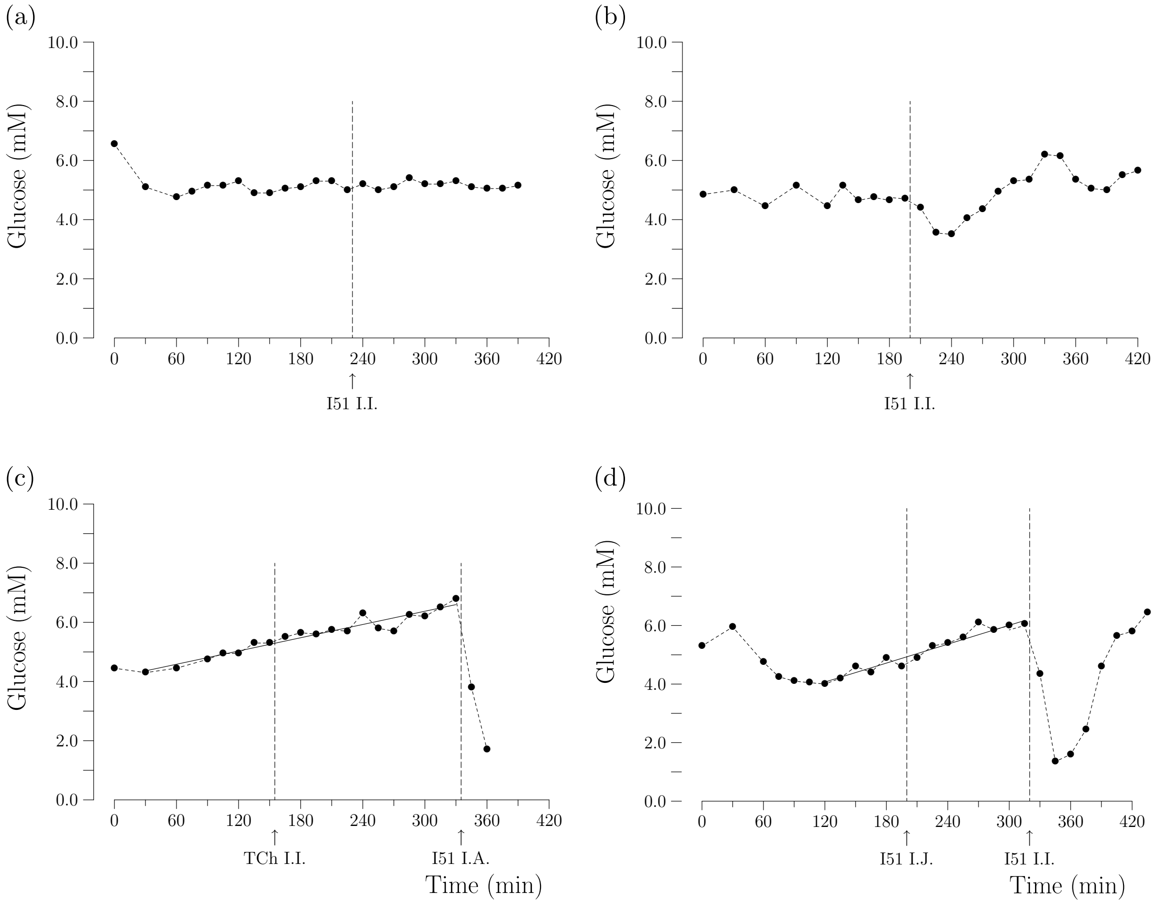

Fig. 4. Representative experiments from 4 different animals showing blood glucose concentration (mM) after the stated intestinal infusions. (a) & (b) 0.7 mg (0.12 μmol) insulin infused intra-ileally (I51 I.I.) (c) Control intra-ileal infusion of 20 mM Na taurocholate (TCh) showing line of best fit for hyperglycaemic increase with $R^2 = 93.4\%, P_{slope} < 0.001$, after which responsiveness was confirmed by I.A. injection of 50 μg I51 (d) Co-infusion of 0.7 mg I51 with 20 mM taurocholate, first, intra-jejunally (I51 I.J.) showing line of best fit for hyperglycaemic increase with $R^2 = 92.5\%, P_{slope} < 0.001$ and, second, intra-ileally (I51 I.I.).

3.4. Intestinal infusions of cholyl-insulins

Intra-ileal infusions of the B^{29}-Lys-cholyl and B^1-Phe-cholyl conjugates of insulin at a dose equimolar to that of insulin (0.8 mg) dissolved in 1.0 mL of the phosphate or citrate/phosphate buffered saline were then undertaken to determine the extent to which they were absorbed across the ileal mucosa. As shown by the representative experiment in Fig. 5a and normalised mean results in Fig. 6, B^{29}-Lys-cholyl-insulin infused intra-ileally in 7 animals was without a significant effect on blood glucose levels ($P = 0.12$, Table 3). In these experiments, the responsiveness of the animal was always confirmed by I.A. injection of conjugate at the end of the experiment.

Table 3

Cumulative changes in blood glucose concentration (mM) after intestinal infusions

Infusate	n	mean ± S.E.M.	P
0.7 mg Insulin I.I.	11	-2.2 ± 0.9 mM	0.034
20 mM TCh I.I.	6	-0.05 ± 0.8 mM	0.96
0.7 mg Insulin + TCh I.I.	6	-18.3 ± 4.2 mM	0.01
0.7 mg Insulin + TCh I.J.	6	+2.1 ± 1.22 mM	0.10
0.8 mg B^{29}-Lys-cholyl-insulin I.I.	7	-3.3 ± 1.8 mM	0.12
0.8 mg B^{1}-Phe-cholyl-insulin I.I.	7	-11.9 ± 1.1 mM	0.001
0.8 mg B^{1}-Phe-cholyl-insulin + TCh I.I.	6	+0.4 ± 0.9 mM	0.65
0.8 mg B^{1}-Phe-cholyl-insulin I.J.	5	+1.9 ± 0.9 mM	0.10

Cumulative change in blood glucose concentration as mean ± S.E.M. with - denoting hypoglycaemia and + denoting hyperglycaemia. I.J. intra-jejunal, I.I. intra-ileal, TCh 20 mM taurocholate. P values are for t-test against zero.

By contrast, B^{1}-Phe-cholyl-insulin infusion resulted in a marked hypoglycaemic response which was evident by 30 min after infusion and lasted for 3 h or more (Figs. 5b & 6) and the cumulative hypoglycaemic response of -11.9 ± 1.1 mM for the 7 animals tested was highly significant (P = 0.001, Table 3). This cumulative response may, however, may be an underestimate since, as shown in Fig. 5b, there is an indication that the hypoglycaemic response became evident against a trend to progressive hyperglycaemia. In the experiments, there was no discernible effect of the addition of citrate to the phosphate buffered saline. The dose-dependance of the hypoglycaemic response was further investigated in groups of 5 animals. At a 0.4 mg dose, the cumulative hypoglycaemic response was -2.9 ± 0.7 mM while, at 1.6 mg, it was -6.9 ± 1.0 mM. Both were significantly lower than the response to 0.8 mg ($P < 0.01$), indicating that 0.8 mg carried in 1.0 mL saline was close to the optimal dose. The process by which B^{1}-Phe-cholyl-insulin was absorbed from the ileum is indicated by the results of the experiments involving co-infusion of the conjugate with 20 mM taurocholate (Figs. 5c & 6) and the experiments in which the conjugate was infused into the jejunum (Figs. 5d & 6). In each case, the hypoglycaemic response was absent ($P > 0.10$, Table 3) which is consistent with the uptake of B^{1}-Phe-cholyl-insulin by the ileal bile salt transporters.

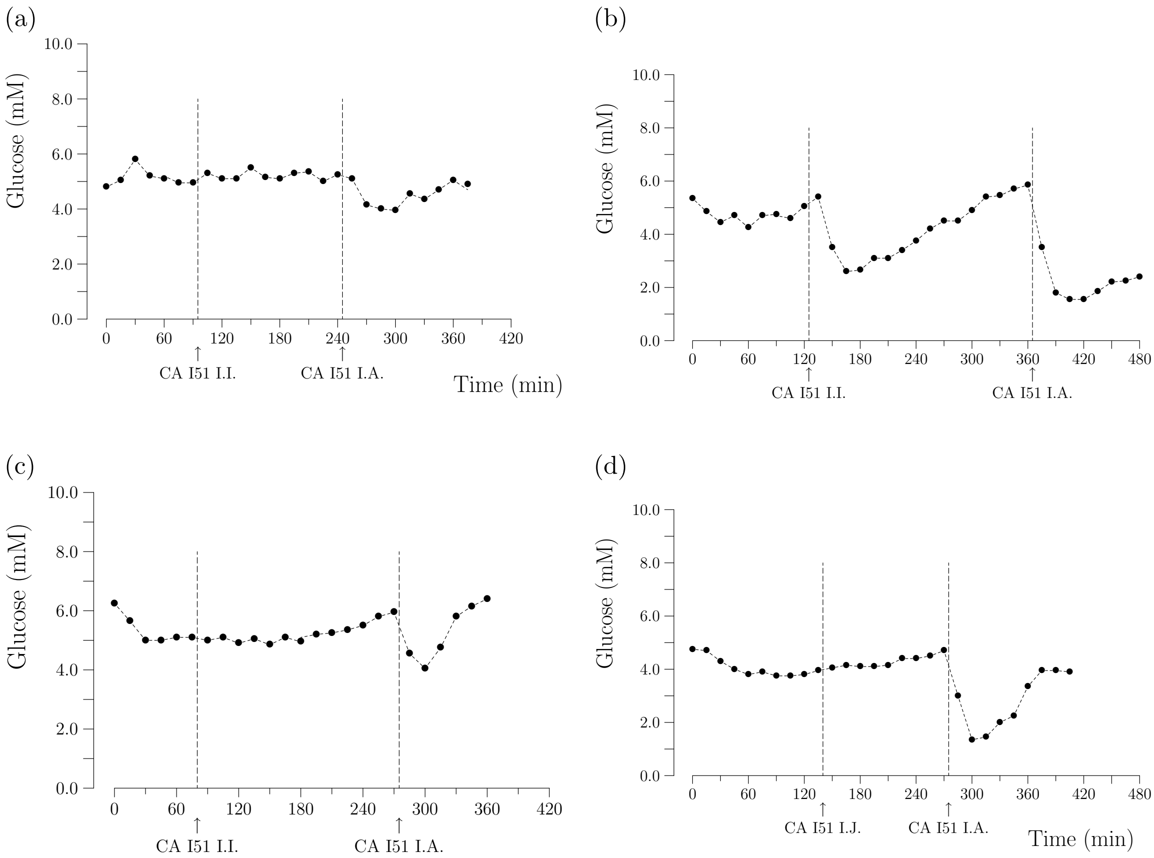

Fig. 5. Blood glucose levels from 4 different experiments in response (a) intra-ileal infusion of 0.8 mg B^{29}-Lys-cholyl-insulin (CA I51 I.I.) followed by an I.A. dose of 3.1 μg cholyl-insulin (CA I51 I.A.) (b) intra-ileal infusion of 0.8 mg B^{1}-Phe-cholyl-insulin (CA I51 I.I.) followed by an I.A. dose of 80 μg cholyl insulin (CA I51 I.A.) (c) intra-ileal infusion of 0.8 mg B^{1}-Phe-cholyl-insulin in 20 mM taurocholate (CA I51 I.I.) followed by I.A. dose of 3.5 μg to test responsiveness (CA I51 I.A.) (d) intra-jejunal infusion of 0.8 mg B^{1}-Phe-cholyl-insulin (CA I51 I.J.) followed by I.A. dose of 35 μg to test responsiveness (CA I51 I.A.).

3.5. Histology of small intestinal mucosa

The results shown in Table 3 are based on experiments in which the small intestinal mucosa showed a normal histological appearance in which the mucosa and villi were intact (Fig. 7a). This was also the case when the infusate contained 20 mM taurocholate (Fig. 7b).

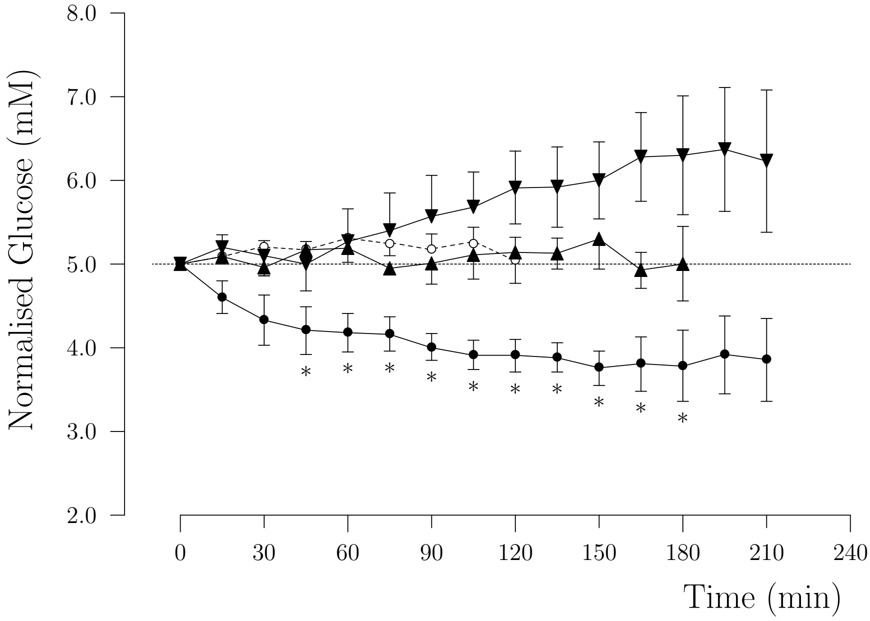

Fig. 6. Mean ± S.E.M. of blood glucose values normalised to a baseline level of 5.0 mM shown by dotted horizontal line in response to: ●-● 0.8 mg (0.12 μmol) B[1]-Phe-cholyl-insulin infused intra-ileally ($n = 7$), ▲-▲ 0.8 mg B[1]-Phe-cholyl-insulin with 20 mM taurocholate infused intra-ileally ($n = 6$), ○-○ 0.8 mg B[1]-Phe-cholyl-insulin infused intra-jejunally ($n = 5$), and ▼-▼ 0.8 mg B[29]-Lys-cholyl-insulin infused intra-ileally ($n = 7$). Significant reduction from 5.0 mM is denoted by * ($P < 0.05$); otherwise there was no significant difference ($P > 0.05$).

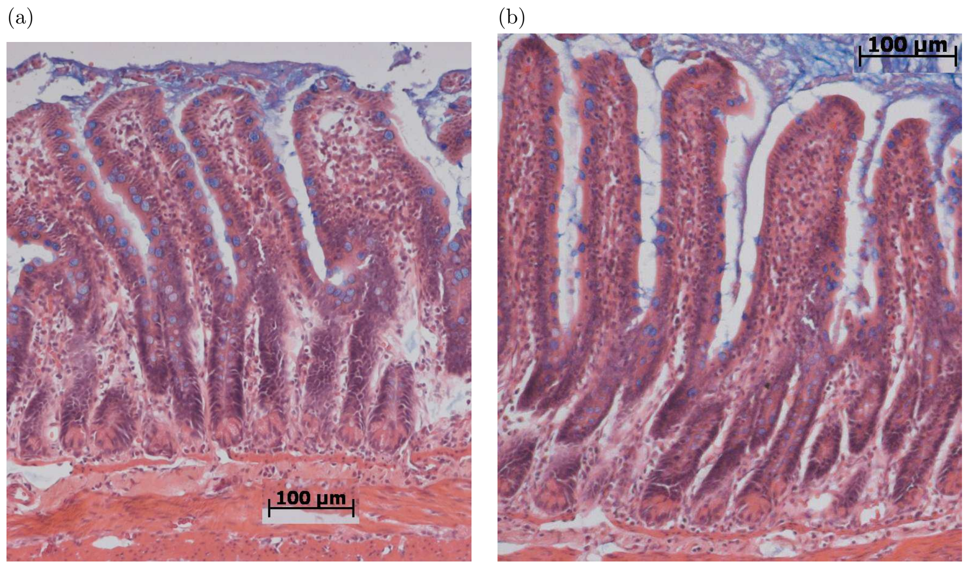

Fig. 7. Normal histology of ileal mucosa after experiments involving infusion of (a) 0.8 mg B[1]-Phe-cholyl-insulin in phosphate saline and (b) 0.8 mg B[1]-Phe-cholyl-insulin in phosphate saline and 20 mM taurocholate. The lumen is at the top and the staining was haematoxylin/eosin and alcian blue.

4. Discussion

The major result from the present study is that conjugation of insulin at the free NH_2 group at the B^1-Phe position with the -COOH group at the C^{24} position of cholic acid to form B^1-Phe-cholyl-insulin promotes absorption from the ileum, while B^{29}-Lys-cholyl-insulin showed no indication of being absorbed (Figs. 5a, 5b & 6, Table 3). This result was obtained in experiments in which the animals were shown to maintain a good physiological condition throughout from their well maintained arterial blood pressure and from the integrity of the ileal mucosa (Figs. 7a & b). As a precautionary measure, pyloric ligation was undertaken to prevent the passage of chyme into the duodenum to avoid the release of gastro-intestinal hormones *viz.* gastrin, cholecystokinin, secretin and gastric inhibitory polypeptide, which have an insulinotropic action [87, 88, 89]. Bilateral vagotomy was also undertaken since the vagus nerves stimulate insulin release [90]. Sometimes, there was the recurring feature of progressive hyperglycaemia which commenced after 3-4 h, particularly in experiments which did not show a hypoglycaemic response *viz.* the control experiments (Fig. 1), infusion of 20 mM taurocholate alone (Fig. 4c) jejunal infusion experiments (Fig. 4d) and ileal infusion of B^{29}-Lys-cholyl-insulin (Fig. 6). The reason is unlikely to be due to the anaesthetic since this only causes a transient hyperglycaemia which subsides by 30 min post-injection [94], though it may account for the initial settling of the blood glucose concentration seen in many experiments *e.g.* Fig. 1. The most likely cause of the delayed hyperglycaemia is surgery-induced ACTH release leading to increased systemic cortisol levels. Since cortisol acts genomically [95], its effect in stimulating gluconeogenesis and antagonising insulin-stimulated glucose uptake [96, 97] requires several hours to take effect after which blood glucose levels would increase unless exogenous insulin was administered.

Systemic injections of natural insulin at the standard molar doses of 2 nmol kg^{-1} and 20 nmol kg^{-1} evoked marked hypoglycaemic responses which were especially prolonged at the higher dose (Fig. 2a) and, on a dose per weight basis, are consistent with previously published data in rat [91, 26], dog [29] and man [92]. This was in contrast to the insulin decapeptide and its cholyl conjugate which, even at 500 times the standard insulin molar dose, showed no evidence of biological activity (Fig. 2b) and is inconsistent with the report that the insulin decapeptide possessed 77% of insulin activity [81]. This was based on the promotion of glycogenesis in adipocytes *in vitro* and highlights the difficulty in relating *in vitro* results to those from the intact animal in which the liver and skeletal muscles have major rôles in glucose uptake. By contrast, both the insulin conjugates, B^{29}-Lys-cholyl-insulin and B^1-Phe-cholyl-insulin, were shown to be biologically active in that, when injected systemically, they caused a hypoglycaemic response which, at the lower standard dose, amounted to some 40% of the insulin response, with a more marked response of extended duration at the higher standard dose (Figs. 2c & d, Table 2). The only previous reports concerning cholyl-insulins relate to systemic injections of B^{29}-Lys-deoxycholyl-insulin which showed a slower onset and longer duration of action than insulin [91] and sub-cutaneous injections of B^{29}-Lys-lithocholyl-insulin which had an enhanced duration of action [93].

For intra-ileal infusions of insulin, there was minimal absorption as shown by an absence of a response or only a small transient hypoglycaemic dip of about 1 mM (Figs. 4a & b) which is consistent with previous studies (Introduction). This result was unaffected by the inclusion of citrate as a Zn^{2+} chelator in the infusion saline, which excludes hexamer formation as a reason for non-absorption of insulin [83]. There was, however, a very dramatic change when insulin was co-infused with 20 mM taurocholate in that a very marked and prolonged hypoglycaemic response was generated, indicating that appreciable amounts of insulin has been absorbed across the ileal mucosa, though this did not occur with co-infusion into the jejunum (Figs. 3 & 4d, Table 3). This facilitatory action of bile salts on insulin absorption is well documented [29, 34, 41, 53, 54, 55, 56], though the absence of response on jejunal infusion has not previously been noted. The reasons for this difference may stem from the presence in the ileum of Peyer's patches, M cells or bile salt transporters. In the event that the bile salt transporters are indeed involved, this might be explained by the hydrophobic attraction of insulin to the bile salt, which would then be taken up by the bile salt transporters. Irrespective of the actual mechanism, the facilitatory action of residual bile salts may provide the explanation for those cases of ileal infusion of insulin alone in which a small hypoglycaemic response in response was recorded (Fig. 4b).

While intra-ileal infusion of B^{29}-Lys-cholyl-insulin was without effect, there was an unequivocal hypoglycaemic response to infusion of B^1-Phe-cholyl-insulin (Fig. 6, Table 3), which is consistent with the absorption of the B^1-Phe-cholyl-insulin into the systemic circulation to promote the uptake of glucose by hepatic and muscle cells. While the absence of absorption of B^{29}-Lys-cholyl-insulin is in part disappointing, these experiments are useful for two reasons. The two conjugates are essentially identical chemically and an absence of an effect with the B^{29}-Lys conjugate supports the conclusion that the B^1-Phe conjugate was not being absorbed across the ileal mucosa by passive diffusion or endocytosis. It also confirms that B^1-Phe-cholyl-insulin was not acting through the release of insulinotropic hormones from the ileum. Since the hypoglycaemic response was abolished by co-infusion with 20 mM taurocholate into the ileum (Figs. 5c & 6) and since the hypoglycaemic response was absent on infusion into the jejunum (Figs. 5d & 6), this would be consistent with absorption of the B^1-Phe-cholyl-insulin through the bile salt transporters which are specific to the ileum [74]. This is plausible since insulin has an overall negative charge [46], thus providing an anionic substrate for the bile salt transporters which are Na^+ dependent [98].

While rather approximate, there is an indication from a comparison of the intra-ileal and intra-arterial cumulative hypoglycaemic responses (Fig. 5b) that the uptake of B^1-Phe-cholyl-insulin may amount to some 5-10% of the intra-ileal dose. However, this may prove to be an underestimate given that the presumed baseline was sometimes equivocal when the response coincided with the onset the delayed hyperglycaemia *e.g.* Fig. 5b. These experiments were undertaken in normal animals after an overnight fast which was necessary to allow emptying of the gastro-intestinal tract: the alternative of using fed animals would, of necessity, involve additional disturbance to small intestine necessitated by the flushing out of the compacted intestinal contents. During the fast, our animals did have access to a sucrose drink which may be surmised to cause endogenous insulin release in order to regulate their blood glucose levels. The fact that B^1-Phe-cholyl-insulin did cause sustained hypoglycaemic responses from normoglycaemic levels in our animals (Fig. 6) may be taken as an encouraging sign for the next stage which would be to determine its effectiveness against uncontrolled hyperglycaemia in diabetic animals without endogenous insulin. Subsequently, it would then need to be established that the result is transferable across the species barrier with the ultimate challenge being in human subjects. In establishing that B^1-Phe-cholyl-insulin was taken up by the ileal bile salt transporters, the uptake was shown to be inhibited by co-infusion of 20 mM taurocholate (Fig. 6), which introduces a potential complication in that, in the normally functioning small intestine, endogenous bile salts would reduce the uptake of B^1-Phe-cholyl-insulin due to competition for sites on the bile salt transporters. However, the rat is unusual in that it releases bile continuously into the duodenum due to the absence of a gall bladder whereas, in man, bile is stored in the gall bladder and is released upon entry of lipids into the duodenum to cause CCK release which is the main effector of gall bladder contraction [99]. This means that delivery of cholyl-insulin in protective capsules prior to a meal could still be a feasible means of promoting absorption by the ileal mucosa. At present, there are 13 initiatives underway using encapsulated insulin, insulin nanoparticles and B^{29}-PEG insulin to enhance the uptake of natural insulin by the intestinal mucosa [38]. Given the toxicity issues and the tendency for initiatives to be abandoned at an early stage, the outlook for an oral insulin formulation is by no means certain [42]. So, it may be timely to consider a new approach in the form of a cholyl-insulin formulation by which absorption is achieved by secondary active transport rather than by passive diffusion and is not dependent upon protease inhibitors or permeation enhancers (Table 1). This may constitute a more productive avenue for further development.

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